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Article

Genotype-Specific Changes in Plant Health Indicators in Buckwheat (*Fagopyrum esculentum*) Cultivars

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Abstract: This study investigated changes in relative indices of leaf chlorophyll (Chl), flavonols (Flav), anthocyanins (Ant), and the nitrogen balance index (NBI) in four buckwheat (*Fagopyrum esculentum*) cultivars through different developmental stages. Measurements were performed using a Dualex optical sensor. Cultivar- and time-dependent differences were assessed by ANOVA with Tukey's HSD post hoc test, while PCA was used to explore multivariate patterns among traits and cultivars. Chl and NBI increased from early weeks, reflecting canopy development and nitrogen assimilation, before gradually decreasing towards the end of the vegetation period. Significant cultivar specific effects occurred at selected time points. Populacija B.T. showed higher Chl (week 5) and elevated NBI (weeks 5 and 7), indicating enhanced nitrogen status and photosynthetic capacity. Darja 1 exhibited significantly higher Ant in weeks 5, 8, and 10, suggesting greater activation of secondary metabolism. Flavonol levels remained relatively stable among cultivars, with minimal differences observed only in week 2. PCA confirmed clear cultivar differentiation driven primarily by Chl, Ant, and NBI. Overall, non-destructive measurements enabled clear differentiation of physiological traits among buckwheat cultivars, supporting its application in breeding, agronomy, and *in vivo* crop assessment.

Keywords: *Dualex; chlorophyll; flavonol; anthocyanin; NBI.*

1. Introduction

Common buckwheat (*Fagopyrum esculentum* Moench) belongs to the family *Polygonaceae* and is a fast-growing pseudo cereal of considerable importance in both human nutrition and agricultural practice, due to its nutritional and medicinal properties. Originating from the northeastern regions of Asia, this plant has been cultivated worldwide for centuries, particularly valued for its ability to thrive on less fertile soils where many other crops cannot succeed [1] .Buckwheat is an alternative crop that is suitable for growing in an organic production system, thanks to its high adaptability to a wide range of different agroecological conditions, which is why it can help overcome the negative impacts of climate change [2]. Buckwheat's resilience, nutritional value, and antioxidant properties make it a promising nutritionally reach food for the future. Importance in modern agriculture of this crop is mainly related to its use as components of functional food based on gluten-free nature, environmental sustainability, nutritional value, rich in essential amino acids, antioxidants, and bioactive compounds like rutin and quercetin [3,4]. Primarily it is cultivated for its grain which is processed into flour and a variety of food products such as biscuits, porridges, and soups buckwheat used in human nutrition [5]. Owing to its diverse phytochemical composition, interest in understanding its physiological responses and metabolic characteristics has increased substantially in recent years.

Due to the increasing demand for food sources rich in biologically valuable compounds, the application of non-destructive, pre-screening methods has become highly relevant in sustainable agriculture. Such approaches enable in vivo monitoring of leaf biochemical parameters, such as the relative contents of photosynthetic pigments and secondary metabolites, allowing important insights into plant physiological status, nutrient balance, and adaptive responses, and helping in choosing accurate harvest time. When applied across developmental stages and cultivars, these measurements provide a powerful basis for understanding growth dynamics, identifying stress resilience, and supporting targeted breeding and agronomic optimisation [6,7]. In this context, the measurement of key pigments and secondary metabolites including flavanols, anthocyanins, and chlorophyll in buckwheat leaves gives insights into the plant's physiological status. Use of sensors, such as Dualex™ Cartelat et al., 2004 [8] offers a non-invasive method to simultaneously examine relative indices of chlorophyll (Chl), flavonols (Flav), anthocyanins (Ant) and the Nitrogen Balance Index (NBI) [9]. Flav and Anth, compounds with strong antioxidant properties, have roles in stress tolerance, photoprotection, and overall metabolic regulation. Their accumulation often reflects the plant's response to environmental conditions, making them indicators of adaptive potential and biochemical activity. Chl content and NBI index, meanwhile, provides insights for plants photosynthetic efficiency and vitality, and nitrogen supply.

The purpose of this study was to investigate relative indices of chlorophyll, epidermal flavonols and anthocyanins in four buckwheat (*Fagopyrum esculentum* Moench) cultivars during different developmental stages using non-destructive measurement with Dualex® Scientific.

2. Materials and Methods

2.1. Experimental design

The experiment was conducted in 2018 at an open-field experimental site in Nenadić (Sombor, Serbia), located near the Toplana area (coordinates 45.748572° N, 18.136816° E), according to complete randomized design in 4 replications. The soil belongs to the chernozem type, and the region is characterized by a temperate continental climate suitable for buckwheat cultivation. Prior to establishing the experiment, pepper (*Capsicum annuum* L.) served as the preceding crop. Standard autumn tillage was performed, followed by the application of 250 kg ha^{-1} of NPK fertilizer in autumn 2018. In early spring, an additional 150 kg ha^{-1} of urea (46% N) was applied to support vegetative development.

Four buckwheat cultivars were included in the experiment: (1) Oberon, (2) B. Petrovac exp. 1, (3) Darja 1, and (4) Populacija B.T. Seeds were sown manually in 3-m-long rows, with 25 cm inter-row spacing and 15 cm spacing between plants within the row. The experimental layout followed standard field cultivation practices for buckwheat, and uniform agronomic management was applied across all treatments, including weed control. No additional fertilization was performed after the spring nitrogen application. Plants were monitored throughout the growing season, and measurements of physiological indices were conducted as explained below.

2.2. Physiological non-destructive measurements

Indices of chlorophyll (Chl), epidermal flavonols (Flav) and their ratio, NBI as well as antocyanines (Anth) were measured in vivo non-destructively with the Dualex sensor (Force-A, Orsay, France). Measurements were made on 7 uniform, fully developed and sun-exposed leaves of the buckwheat plants per each replication, each fourteenth day starting from sowing in soil. The measurement period covered ten distinct developmental stages ranging from the seedling phase (week 1) to physiological maturity (week 10). Based on the known phenology of *Fagopyrum esculentum*, weeks 1 to 3 which correspond to vegetative growth, weeks 4 to 6 which correspond to flowering and early seed formation, and weeks 7 to 10 leading to seed maturation and leaf physiological maturity.

The Chl-content estimation is based on a difference in the transmission of two distinct wavelengths: visible (VIS) (650 nm) and near-infrared (NIR) (710 nm). The epidermal flavonols content is determined by comparing the absorbance at ultraviolet A (UVA) (375 nm) and 650 nm. Both wavelengths excite Chl fluorescence, but only UVA is affected by flavonols. The difference in Chl fluorescence measured at 710 nm is directly proportional to the amount of epidermal flavonols. The nitrogen balance index (NBI) is calculated as the Chl/Flav ratio [8].

2.3. Statistical analyses

All statistical analyses were performed using STATISTICA version 14.0.0.15 (TIBCO Software Inc., Palo Alto, CA, USA). Measurements at each sampling week were obtained from independent plants; therefore, weeks were considered developmental stages rather than experimental treatments. Cultivar effects were assessed by one-way analysis of variance (ANOVA) separately for each sampling week, followed by Tukey's Honestly Significant Difference (HSD) post hoc test at a significance level of $\alpha = 0.05$. Multivariate relationships among measured leaf traits (Chl, Flav, Anth, and NBI) across sampling weeks were explored by principal component analysis (PCA) performed on a correlation matrix

3. Results

3.1. Indice of chlorophyl

Relative chlorophyll indices in the four buckwheat cultivars generally showed no significant variation across the examined developmental stages. During the first vegetative stages, from weeks 1 to 3, Chl increased slightly from 20.02 to 21.44 to 29.28 to 29.35, respectively. The pre-flowering phase (Week 3 to 4) exhibited the highest Chl values across all cultivars, being the highest 31.05 at 32.88. As buckwheat entered the early and peak flowering phases Chl values decreased from week 4 to 6. In week 5, a significant difference was observed for Populacija B.T., which showed significantly higher chlorophyll values compared to the other cultivars. A similar pattern was observed for Darja 1, which also exhibited higher chlorophyll content than the remaining cultivars at this stage. During late flowering and early seed set, in week 7, Darja 1 showed a significant Chl increase (34.10). In the last weeks (8-10), Chl consistently decreased, indicating progressive leaf aging and nitrogen remobilization typical for senescing plants.

3.2. Indice of epidermal flavonol content (Flav)

Flavonol levels remained relatively stable throughout the experiment, showing minor fluctuations across developmental phases. During vegetative growth (weeks 1–3), values ranged from 1.85 (Populacija B.T, week 1.) to 2.07 (Darja, week 2), with statistically significant value for this cultivar. During flowering and early seed formation phase (week 4 to 6), Flav indices did not show any statistically significant differences through each examined week. A similar trend persisted during the seed development and maturation phases (weeks 7 to 10), where Flav levels continued to show stable values without significant genotype-dependent variation.

3.3. Anthocyanin content (Ant)

Throughout the experiment, the Ant of buckwheat leaves showed characteristic increases during the different vegetative phases, with Darja 1 consistently exhibiting higher values compared with the other three cultivars at several measurement points. During the vegetative phase (weeks 1 to 3), Ant values were low and similar across cultivars (ranging between 0.10 to 0.18). A significant increase occurred at the beginning of flowering (week 5), where Ant for Darja 1 was 0.09, compared with a range of 0.07 to 0.08 for the other 3 cultivars. This cultivar specific increase was again evident during the seed development stage (week 8), when Ant in Darja 1 increased to 0.13, while the other cultivars showed the same value of 0.10. The strongest difference was recorded at physiological maturity stage (week 10), with Darja 1 reaching 0.17, in contrast to 0.12 to 0.15 in the other cultivars. These higher values of Ant in Darja 1 reflect a cultivar-specific tendency for anthocyanin accumulation during flowering and physiological maturity compared with Oberon, B. Petrovac exp. 1, and Populacija B.T.

3.4. Nitrogen Balance Index (NBI) in relation to physiological phases

Throughout the experiment, the nitrogen balance index (NBI) of buckwheat leaves followed the same developmental pattern as chlorophyll (Chl), with higher values during vegetative growth and early flowering, followed by decrease during seed development and maturation. During the vegetative phase (weeks 1 to 3), NBI increased from 10.54 to 11.43 (week 1), 11.64 to 12.59 (week 2) and from 14.18 to 14.76 (week 3) across all cultivars. Highest values were observed from late vegetative to early flowering developmental phase (week 4), when values for all cultivars were up to 17.16. Clear cultivar differences then emerged during reproductive development: in week 5, Populacija B.T. showed the highest NBI (15.12), while the remaining cultivars ranged from 13.17 to 14.33. A second significant increase was observed for Darja 1 in week 7, where NBI reached 18.82. During the seed development and maturation phases (weeks 8 to 10), NBI progressively decreased to 13.37 for cultivar Populacija B.T. in week 10.

Table 1. Weekly genotype-specific variation in plant health indicators (chlorophyll, flavonols, anthocyanins and NBI) in buckwheat (*Fagopyrum esculentum*) cultivars (mean ± SD).

Week	Cultivar	Chl	Flav	Ant	NBI
1	1	21.43 ± 2.66 a	1.89 ± 0.11 a	0.17 ± 0.02 a	11.38 ± 1.46 a
1	2	20.02 ± 2.95 a	1.90 ± 0.10 a	0.18 ± 0.03 a	10.54 ± 1.53 a
1	3	21.44 ± 2.60 a	1.91 ± 0.09 a	0.17 ± 0.02 a	11.23 ± 1.50 a
1	4	21.05 ± 2.83 a	1.85 ± 0.11 a	0.17 ± 0.03 a	11.43 ± 1.82 a
2	1	23.64 ± 3.13 a	2.04 ± 0.08 a	0.15 ± 0.02 a	11.64 ± 1.77 a
2	2	24.20 ± 3.84 a	2.00 ± 0.06 a	0.14 ± 0.02 a	12.09 ± 1.96 a
2	3	25.93 ± 3.65 a	2.07 ± 0.09 b	0.14 ± 0.02 a	12.56 ± 1.82 a
2	4	25.70 ± 3.35 a	2.05 ± 0.10 ab	0.14 ± 0.02 a	12.59 ± 1.80 a
3	1	29.35 ± 4.20 a	2.00 ± 0.10 a	0.10 ± 0.02 a	14.76 ± 2.32 a
3	2	29.15 ± 4.85 a	2.04 ± 0.09 a	0.10 ± 0.02 a	14.33 ± 2.50 a
3	3	29.28 ± 4.63 a	2.03 ± 0.11 a	0.12 ± 0.03 a	14.43 ± 2.42 a
3	4	28.74 ± 6.35 a	2.00 ± 0.20 a	0.11 ± 0.02 a	14.18 ± 3.18 a
4	1	32.88 ± 6.47 a	1.92 ± 0.21 a	0.07 ± 0.03 a	17.16 ± 3.09 a
4	2	32.67 ± 6.13 a	1.95 ± 0.17 a	0.08 ± 0.03 a	16.80 ± 3.21 a
4	3	31.05 ± 6.49 a	1.92 ± 0.33 a	0.09 ± 0.03 a	16.10 ± 2.65 a
4	4	32.40 ± 7.99 a	1.96 ± 0.23 a	0.09 ± 0.03 a	16.48 ± 4.27 a
5	1	25.52 ± 4.47 a	1.81 ± 0.20 a	0.09 ± 0.02 a	14.33 ± 3.44 a
5	2	25.74 ± 3.14 a	1.90 ± 0.11 a	0.09 ± 0.01 a	13.65 ± 2.06 a
5	3	25.26 ± 4.46 a	1.92 ± 28 a	0.09 ± 0.01 a	13.17 ± 1.46 a

5	4	28.66 ± 4.26 b[1]	1.90 ± 0.12 a	0.08 ± 0.02 b	15.12 ± 2.02 b[1]
6	1	27.24 ± 4.14 a	2.02 ± 0.07 a	0.10 ± 0.02 a	13.50 ± 2.15 a
6	2	28.40 ± 5.92 a	1.96 ± 0.19 a	0.09 ± 0.02 a	14.67 ± 3.58 a
6	3	26.81 ± 5.01 a	2.00 ± 0.15 a	0.09 ± 0.02 a	13.46 ± 2.55 a
6	4	27.09 ± 4.83 a	1.99 ± 0,16 a	0.09 ± 0.02 a	13.61 ± 2.51 a
7	1	30.82 ± 4.22 a	1.89 ± 0.11 a	0.07 ± 0.02 a	16.34 ± 2.28 a
7	2	32.26 ± 5.32 a	1.86 ± 0.09 a	0.08 ± 0.02 a	17.40 ± 2.87 a
7	3	34.10 ± 4.33 b[1]	1.83 ± 0.15 a	0.07 ± 0.02 a	18.82 ± 3.06 b[1]
7	4	32.91 ± 4.34 ab	1.86 ± 0.12 a	0.07 ± 0.02 a	17.81 ± 2.61 ab
8	1	27.41 ± 3.47 a	1.87 ± 0.16 a	0.10 ± 0.02 a	14.80 ± 2.55 a
8	2	28.46 ± 5.42 a	1.84 ± 0.21 a	0.10 ± 0.03 a	15.52 ± 2.85 a
8	3	26.87 ± 5.77 a	1.85 ± 0.12 a	0.13 ± 0.04 b	14.59 ± 3.25 a
8	4	29.37 ± 5.35 a	1.91 ± 0.16 a	0.10 ± 0.04 a	15.44 ± 2.96 a
9	1	26.13 ± 7.23 a	1.78 ± 0.47 a	0.11 ± 0.04 a	15.67 ± 4.94 a
9	2	29.94 ± 6.05 a	1.88 ± 0.32 a	0.11 ± 0.04 a	16.38 ± 3.94 a
9	3	27.64 ± 5.15 a	1.94 ± 0.25 a	0.12 ± 0.03 a	14.36 ± 2.29 a
9	4	27.49 ± 6.07 a	1.96 ± 0.15 a	0.12 ± 0.03 a	14.10 ± 3.17 a
10	1	24.69 ± 4.78 a	1.70 ± 0.26 a	0.12 ± 0.02 a	14.85 ± 3.41 a
10	2	24.65 ± 4.36 a	1.76 ± 0.24 a	0.15 ± 0.05 a	14.29 ± 3.34 a
10	3	23.34 ± 4.61 a	1.75 ± 0.34 a	0.17 ± 0.06 b[1]	16.50 ± 18.11 a
10	4	24.41 ± 5.48 a	1.85 ± 0.20 a	0.16 ± 0.05 ab	13.37 ± 3.50 a

[1] Values are presented as mean ± standard deviation (n = 26–28 per cultivar × week combination). Within each week and each parameter, means followed by different letters differ significantly at $P \leq 0.05$ according to Tukey's HSD test

3.5. PCA analysis

Principal component analysis (PCA) performed on standardized Dualex-derived traits revealed a clear multivariate separation among the four buckwheat cultivars (Figure 1). The first two principal components captured most of the total variance, with PC1 explaining approximately 65.8% and PC2 28.6%, together accounting for over 94% of the overall variability. In the PCA score plot (Figure 1.), cultivars are distinctly separated along both principal axes, indicating pronounced genotype-specific physiological profiles that integrate pigment- and nitrogen-related traits across sampling weeks. This clear spatial separation confirms that cultivar differentiation is not driven by a single trait, but by coordinated multivariate responses.

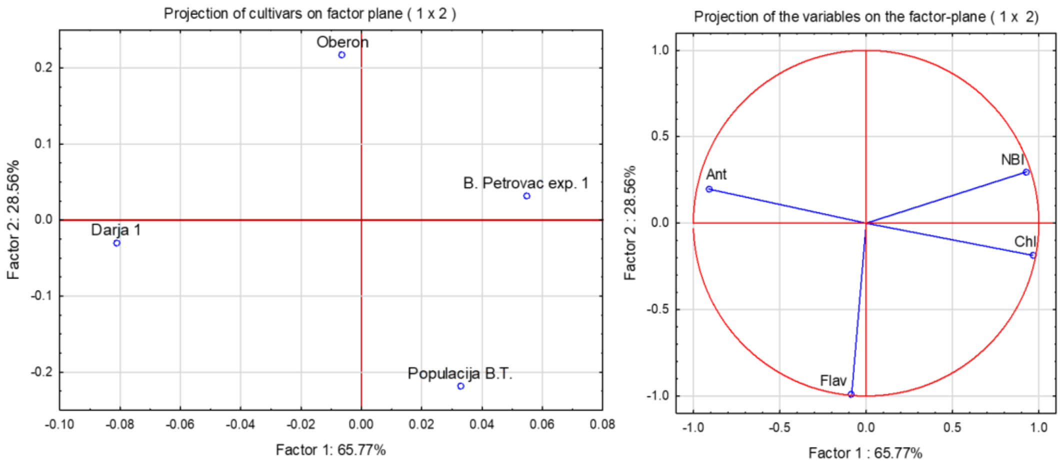

Figure 1. PCA of standardized Dualex-derived leaf traits (**a**) Score plot showing cultivar centroids (mean PC1–PC2 scores calculated from the full dataset); (**b**) Loading plot (correlation circle) indicating trait contributions to PC1 and PC2.

Separation along PC1 is primarily associated with chlorophyll- and nitrogen-related indices (Chl and NBI), as indicated by their strong and aligned loadings in the correlation circle. Cultivars with higher PC1 scores exhibit consistently elevated Chl and NBI values, reflecting enhanced photosynthetic capacity and nitrogen status. These traits were also identified as significant contributors in one-way ANOVA at multiple sampling weeks, supporting their dominant role in cultivar differentiation.

The PC2 axis is mainly driven by anthocyanin-related variation, as shown by the near-orthogonal orientation of the anthocyanin loading relative to Chl and NBI vectors. This suggests that PC2 captures cultivar-specific pigment responses, likely associated with developmental stage, stress sensitivity, or phenological differences, which were detected by ANOVA at selected time points.

Overall, while ANOVA identified cultivar-dependent differences in individual traits at specific sampling weeks, PCA integrated these effects into stable and interpretable multivariate physiological patterns, revealing coherent genotype-specific signatures across the growing period, dominated by chlorophyll–nitrogen relationships along PC1 and pigment-specific (anthocyanin) responses along PC2.

4. Discussion

Plant health indicators investigations among buckwheat cultivars relate to better understanding of the most appropriate sowing time, best harvest time, efficient fertilizer management, as well as yield instability in buckwheat. The limited and unstable cultivation of buckwheat is closely associated with cultivar-dependent physiological traits [10, 11, 12]. It is mostly recognized during different developmental stages such as flowering and physiological maturity, when yield losses due to flower abortion and nitorgen-related imbalance frequently occur [13]. The content of Chl, is generally used as an indicator of plant nitrogen status, given the central role of nitrogen in chlorophyll synthesis, whereas contents of flavonols and anthocyanins reflect the activity of the plant antioxidant system [9]. These compounds have a protective role by scavenging reactive oxygen species, chelating metal ions, and shielding photosynthetic tissues from oxidative damage.

Our results demonstrate variation in leaf pigment indices and NBI in relation to different cultivars, across its developmental stages. The investigation of chlorophyll indices observed in this study, showed slight increase during vegetative and beginning of the flowering period, with significant increase for Darija 1 and Populacija B.T, in the week 7 and week 5, respectively with decreased during flowering and physiological maturity of all examined cultivars. A positive correlation was observed between chlorophyll content and Nitrogen Balance Index (NBI) values, indicating that increased nitrogen availability leads to enhanced chlorophyll synthesis and improved plant nitrogen status. These results are consistent with previously reported developmental stages in buckwheat and other crops assessed using optical sensors [7,8]. Martinez et al. 2022 [9], reported that chlorophyll content in buckwheat closely reflects nitrogen availability and biomass formation, particularly during the transition from vegetative to reproductive growth, explaining the functional relevance of chlorophyll-based indices for evaluating crop physiological status under field conditions. Bojovic et al. (2023) [14] used Dualex to study changes in tomato leaves, where chlorophyll content was closely associated with NBI index through the experiment. In our study, cultivar specific differences in Chl and NBI were highest at flowering stage which is known to have a significant role in determining yield of buckwheat [15]. The consistently higher Chl and NBI values recorded for Populacija B.T. in the mid-flowering stage suggests that this cultivar has a capacity to maintain nitrogen assimilation and photosynthetic activity at level when nitrogen is increasingly redistributed from vegetative tissues to developing reproductive organs [16, 17]. Comparable relationships between Chl and NBI have been reported by Vukelić et al. (2021) [18], who demonstrated that *Trichoderma harzianum* application in tomato induced changes in Chl related parameters and NBI, reflecting a regulated adjustment of primary metabolism under biotic interaction. These findings support the interpretation that relationship of Chl and NBI during reproductive development represents an important component of physiological resilience of plants [19]. This interpretation is further supported by Agati et al. (2015) [19], showed that the opposite responses of Chl and Flav to nitrogen availability, combined in the NBI, can be used as a reliable indirect indicator of plant nitrogen status in different crops. Comparable genotype dependent differences in chlorophyll related indices have been documented by Sytar et al. (2019) [20], who demonstrated that buckwheat genotypes of different origin exhibit distinct pigment dynamics during vegetative and early reproductive phases, consistent with genetically determined physiological divergence rather than environmentally induced short-term responses.

In contrast to Chl and NBI, results for Anth indices were cultivar specific during maturation phase, while Flav indices remained relatively stable across examined cultivars and developmental stages, with only minor genotype specific differences detected early plant growth. This is in agreement with previous results of Domingos and Bilsborrow (2021) [12] who measured antioxidant activity of 2 buckwheat varieties (Bamby and Čebelica over 3 growing seasons) and showed little variation in total antioxidants in seeds, as determined by a colorimetric absorbance method. Moreover, it was indicated that epidermal flavonols function primarily as constitutive ultraviolet screening compounds rather than stress indicators under moderate field conditions which is reported by Sytar et al. (2019) [20] They explained limited variation in Flav indices among buckwheat genotypes during vegetative growth, supporting the interpretation that Flav accumulation represents a general photoprotective characteristic that shows limited variation among genotypes under optimal conditions. Moreover, Agati et al. (2015) [19] demonstrated that Flav accumulation exhibits a strong inverse relationship with nitrogen availability, with limited variation observed under sufficient nitrogen nutrition and its increases occurring primarily under nitrogen deficiency. Genotype Darja 1 had higher Anth indices during flowering, seed development, and physiological maturity. Anth accumulation in buckwheat has been associated with sowing time and developmental regulation and oxidative stress mitigation. Sytar et al. (2019) [20] reported that genotypes with higher Anth indices shown different physiological responses during flowering and in photosynthetic pigment composition together with higher antioxidant levels Similarly, Ullah et al. (2025) [22] demonstrated that increased Anth and Flav contents accompanied reductions in chlorophyll and nitrogen balance index under copper and zinc exposure, reflecting a metabolic shift from primary toward secondary metabolism.

Although the present study was conducted under non stress field conditions, the repeated increment of Anth indices in Darja 1 suggests higher activation potential of phenylpropanoid metabolism during developmental stages of buckwheat genotypes. Such a response may contribute to improved tolerance to endogenous oxidative stress associated with flowering and leaf senescence, supporting that anthocyanin accumulation in buckwheat is not only stress induced but also genetically regulated [23].

5. Conclusions

Pre-screening of biologically active compounds and pigments using optical sensors enables rapid, nondestructive assessment of their relative indices during plant development. Genotype specific measurements help in better understanding of photosynthetic functionality while differences in antioxidant related indices may reflect resilience under variable environmental conditions. The differences observed among buckwheat cultivars in Chl, Flav, Anth indices and NBI highlights the relevance of non-destructive methods for defining genotype specific strategies based on buckwheat growth development. Our results might help in choosing optimal time of the harvest for buckwheat plants due to its maturation stages, as well as for Flav and Anth related indices estimation facilitating selection of cultivars with higher antioxidant potential. Overall, the applied non destructive method use has been proven as effective for distinguishing physiological traits among buckwheat cultivars under field conditions, supporting its application in crop assessment.

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Conflicts of Interest: The authors declare no conflict of interest.

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